# Electronic Supplementary Information

# Antimicrobial properties of nanostructured surfaces – demonstrating the need for a standard testing methodology

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**Table 1** Comparison of assays commonly used to determine bacterial viability after exposure to antimicrobial designs. Assays 1 to 3 evaluate viability of the non-attached cells whereas assays 4 and 5 characterize viability of the cells attached to the surface. Criteria of the comparison include: accuracy with sources of false positive (FP, surface is or is more antimicrobial when it is not in reality) and false negative (FN, surface is not or is less antimicrobial than it is in reality) results; advantages and disadvantages of the assays.

| | **Assay** | **Description** | **Accuracy** | **Advantages** | **Disadvantages** |
|---|---|---|---|---|---|
| **1** | **Colony counting method (spread plate)** | Bacteria are spread on an agar plate and Colony Forming Units (CFU) are counted as an equivalent of viable cells in original suspension | High<br>**FP** cluster of many cells forms CFU (when on tested samples)<br>**FN** cluster of many cells forms CFU (when on control samples) | - Inexpensive<br>- Simple<br>- Standard method | - Time consuming<br>- Big amount of disposable material<br>- Determines only viable cells that able to grow |
| **2** | **Optical Density (OD)** | Turbidity of cell suspension is measured by spectrophotometer and correlated with cell concentration | Low<br>**FP** n/a<br>**FN** (i) Unable to distinguish live from dead cells (ii) Cell clusters block light more efficiently | - Inexpensive<br>- Simple<br>- Fast<br>- Rate of proliferation can be determined | - Spectrophotometer is needed<br>- Unable to distinguish live from dead cells (unless they are lysed) |
| **3** | **Flow Cytometry *live/dead staining*** | Cell suspensions are stained with fluorescent dyes that allow discriminating live from dead cells based on various viability criteria. The samples are analyzed by cytometry, providing cell counts for each subgroup | High<br>**FP** n/a<br>**FN** injured cells scored as viable but not able to proliferate | - Fast<br>- High-throughput when plate loader available<br>- Quantifies total, viable and non-viable number of cells<br>- Provides data regarding cell morphology<br>- Various fluorescent stains available to assess viability (membrane integrity, metabolic activity, membrane polarization etc.) | - Costly instrument<br>- Limitations of fluorescent staining applies |

| | | | | | |
|---|---|---|---|---|---|
| **4** | **Fluorescence Microscope *live/dead staining*** | Cells attached to the surface or in solution are stained with fluorescent kit (see above), and imaged by florescence microscope | High<br>**FP** sample preparation<br>**FN** (i) attached cells lost during surface washing from non-attached cells (ii) already lysed cells are not visible | - Data about cell adhesion<br>- Determines viable and non-viable cells<br>- Surface analysis possible<br>- Various fluorescent stains available to assess viability (membrane integrity, metabolic activity, membrane polarization etc.) | - Costly instrument<br>- Time consuming<br>- Low-throughput<br>- Limitations of fluorescent staining applies |
| **5** | **Scanning Electron Microscope (SEM)** | Cells attached to the surface (or in solution and transferred onto the supporting material) are fixed, dehydrated, dried, covered with thin conductive film, and imaged using electron microscope | High or moderate<br>**FP** incorrect sample preparation<br>**FN** (i) lack of resolution when too low voltage applied or due to the charging effects, (ii) incorrect sample preparation | - Great resolution<br>- Provides in-depth analysis that can speak to antimicrobial mechanisms<br>- Ability to evaluate both non-attached and attached cells onto the surface | - Costly instrumentation is needed (SEM, critical-point dryer, sputtering system)<br>- Complex sample preparation, prone to artifacts<br>- Contrasting requires toxic staining reagents<br>- Time consuming<br>- Difficult to analyze, resulting in high or moderate accuracy<br>- Experience required<br>- Rather not quantitative |

**Supplementary Text 1** Media preparation.

Media and buffers were prepared using deionized water according to the following recipes:

(a) **Phosphate-buffered saline (PBS)** – 1 x PBS contains: 0.001 M $KH_2PO_4$, 0.01 M $Na_2HPO_4$, 0.137 M NaCl, 0.0027 M KCl; pH was adjusted to 7.4.

(b) **Tris-buffered saline (TBS)** – 1 x TBS contains: 50 mM Tris-Cl buffer (pH 7.5), 150 mM NaCl.

(c) **M9 medium** – 1 x M9 contains: 15 g/l $KH_2PO_4$, 33.9 g/l $Na_2HPO_4$, 2.5 g/l NaCl, 5 g/l $NH_4Cl$; pH adjusted to 6.8. M9 medium with glucose contains 20 ml of 20% glucose solution.

(d) **MR 26 medium** – First, prepare reagents A, B, C. Reagent A is 1 M potassium phosphate buffer ($K_2HPO_4$ 115g/l, $KH_2PO_4$ 44.9 g/l; pH adjusted to 6.8). Reagent B is 1 M ammonium succinate (succinic acid 115g/l, pH adjusted to 6.8 with $NH_4OH$). Regent C is a concentrated base that consists of compounds dissolved in $H_2O$ in order as listed in Table S2 below, followed by pH adjustment to 6.8 with $NH_4OH$.

**Table S2** A list of compounds used in preparation of Regent C.

| Compound | Concentration (g/l) |
|---|---|
| $Na_2$-EDTA x 2 $H_2O$ | 11.16 |
| $(NH_4)_6Mo_7O_{24}$ x 4 $H_2O$ | 0.0093 |
| $FeSO_4$ x 7 $H_2O$ | 0.099 |
| “Metals 44” | 50 ml |
| $MgSO_4$ | 14.5 |
| $CaCl_2$ | 2.5 |

“Metals 44” contains the following list of compounds (Table S3) dissolved in 1 l of $H_2O$:

**Table S3** A list of compounds used in preparation of “Metals 44”.

| Compound | Concentration (g/l) |
|---|---|
| $Na_2$-EDTA x 2 $H_2O$ | 6.5 |
| $FeSO_4$ x 7 $H_2O$ | 5.0 |

| | |
|---|---|
| $ZnSO_4$ x 7 $H_2O$ | 10.9 |
| $MnCl_2$ x 4 $H_2O$ | 1.3 |
| $CuSO_4$ x 5 $H_2O$ | 0.392 |
| $CoCl_2$ x 6 $H_20$ | 0.200 |
| $H_3BO_3$ | 0.113 |

Use 20 ml of A. B and C per 1 l of MR26 medium.

(e) **Super Optimal broth with Catabolite repression (SOC)** contains 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose.

**Table S4** Total number of cells suspended in various media and buffers, at initial time (0 h) and after 3 h. The cells were counted by flow cytometry and the values are expressed as cells/µl.

| **Cell type** | **Time (h)** | **Media and Buffers** | | | | |
|---|---|---|---|---|---|---|
| | | **PBS** | **TBS** | **MR26** | **M9 salts** | **M9+glucose** |
| ***E. coli*** | 0 | 1050 | 991 | 1162 | 598 | 680 |
| | 3 | 1031 | 921 | 1152 | 620 | 677 |
| ***R. capsulatus*** | 0 | 890 | 748 | 700 | 1000 | 504 |
| | 3 | 340 | 607 | 646 | 567 | 645 |

**Supplementary text 2** Statistical analysis

Each experiment was conducted with three technical replicates and minimum of three biological replicates. The data were assumed to have a normal distribution and thus an independent t-test, two-tails was performed in order to compare whether the differences between two means were significant at the significance level of 0.05. A statistically significant t-test result is one in which a difference between two groups is unlikely to have occurred because the sample happened to be atypical. Two hypotheses were tested:

1. Is there a significant difference between the antibacterial efficiencies derived from testing in rich medium or nutrient-free buffer?
2. Is there a significant difference between the antibacterial efficiencies assessed by cytometry or colony counting method (plates)? (to validate cytometry suitability to replace plating)

The data are listed below and organized by:

1. Material type – long (sharp) or short (blunt) pillars which, broadly, correspond to two killing mechanisms that occur on these structures, direct piercing and stretch-and-tear, respectively.
2. Organism type – two Gram-negative species tested, *E. coli* and *R. capsulatus.*

Long pillars

| Organism | Method | Rich vs Buffer; t-test results | Significant result |
|---|---|---|---|
| *E. coli* | Cytometry | t(7)=3.040, p=0.019 | yes |
| | Plates | t(4)=2.827, p=0.047 | yes |
| | Microscopy | t(2)=2.279, p=0.150 | no |
| *R. capsulatus* | Cytometry | t(2)=1.000, p=0.423 | no |
| | Plates | t(2)=0.526, p=0.651 | no |
| | Microscopy | t(2)=22.000, p=0.00206 | yes |

| Organism | Method | Cytometry vs Plates; t-test results | Significant result |
|---|---|---|---|
| *E. coli* | Rich | t(3)=2.031, p=0.135 | no |

| | | | |
|---|---|---|---|
| | Buffer | t(4)=7.466, p=0.000072 | yes |
| *R. capsulatus* | Rich | t(6)=0.331, p=0.752 | no |
| | Buffer | t(6)=0.467, p=0.657 | no |

Short pillars:

| Organism | Method | Rich vs Buffer; t-test results | Significant result |
|---|---|---|---|
| *E. coli* | Cytometry | t(6)=2.574, p=0.042 | yes |
| | Plates | t(6)=5.597, p=0.00139 | yes |
| | Microscopy | t(3)=7.606, p=0.00472 | yes |
| *R. capsulatus* | Cytometry | t(4)=1.610, p=0.183 | no |
| | Plates | t(4)=2.216, p=0.078 | no |
| | Microscopy | t(2)=1.080, p=0.393 | no |

| Organism | Method | Cytometry vs Plates; t-test results | Significant result |
|---|---|---|---|
| *E. coli* | Rich | t(5)=1.275, p=0.258 | no |
| | Buffer | t(7)=2.722, p=0.030 | yes |
| *R. capsulatus* | Rich | t(6)=1.270, p=0.251 | no |
| | Buffer | t(3)=2.468, p=0.090 | no |

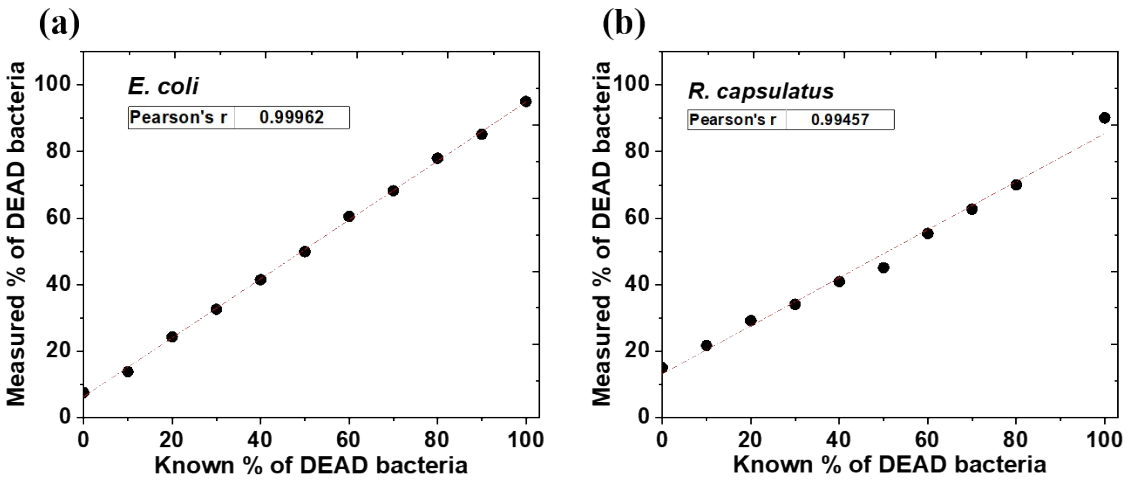



**Figure S1.** Live/dead assay optimization for (a) *E. coli* and (b) *R. capsulatus.* Two cell controls were prepared: (i) live cells, and (ii) dead cells, by subjecting them to heat-inactivation for 30 min at 60 °C. Two controls were mixed at known ratio, stained with SYTO9 and PI fluorescent dyes, and the number of live and dead cells was measured by flow cytometry. The dyes were mixed in the range of 0.8-1.2 of SYTO9:PI ratio. The graphs present data for ratio of 1 which was found as optimal.

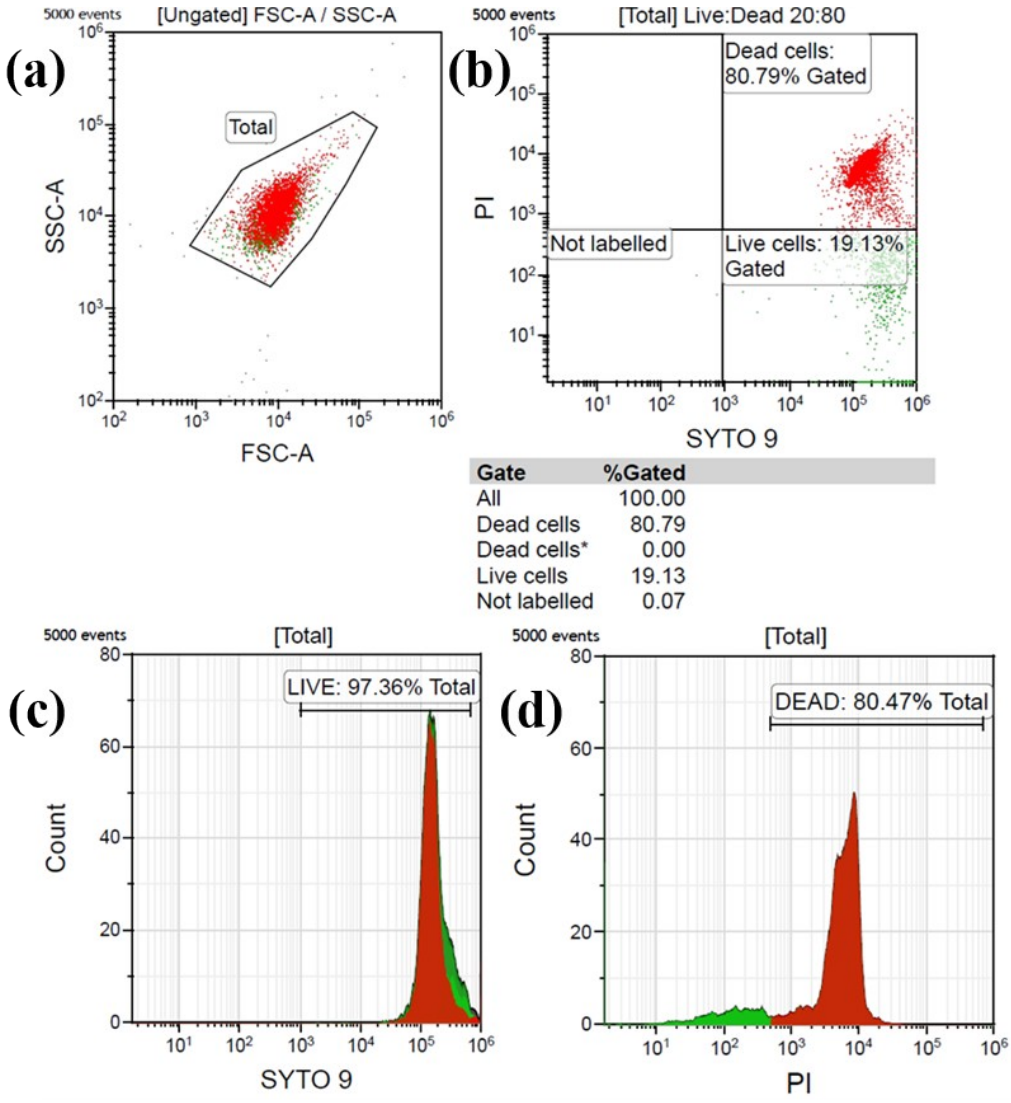



**Figure S2.** Representative cytograms for *E. coli* (cells suspended in TBS). Live and dead cells were mixed at 20:80 ratio, stained with SYTO 9 and PI dyes, and assayed by flow cytometer. (a) SSC (side scattering) versus FSC (forward scattering) cytogram characterizes cells based on their morphology and serves to indicate a population of interest (total population of cells is gated out; *Total*). (b) PI versus SYTO 9 cytogram shows population of live and dead cells (green and red, respectively). The percentage of cells in each population is quantified and listed in a table below the cytogram. (c, d) The number of cells stained by SYTO 9 and PI dyes, respectively. The histogram is just a different graphical representation of the Graph (b).

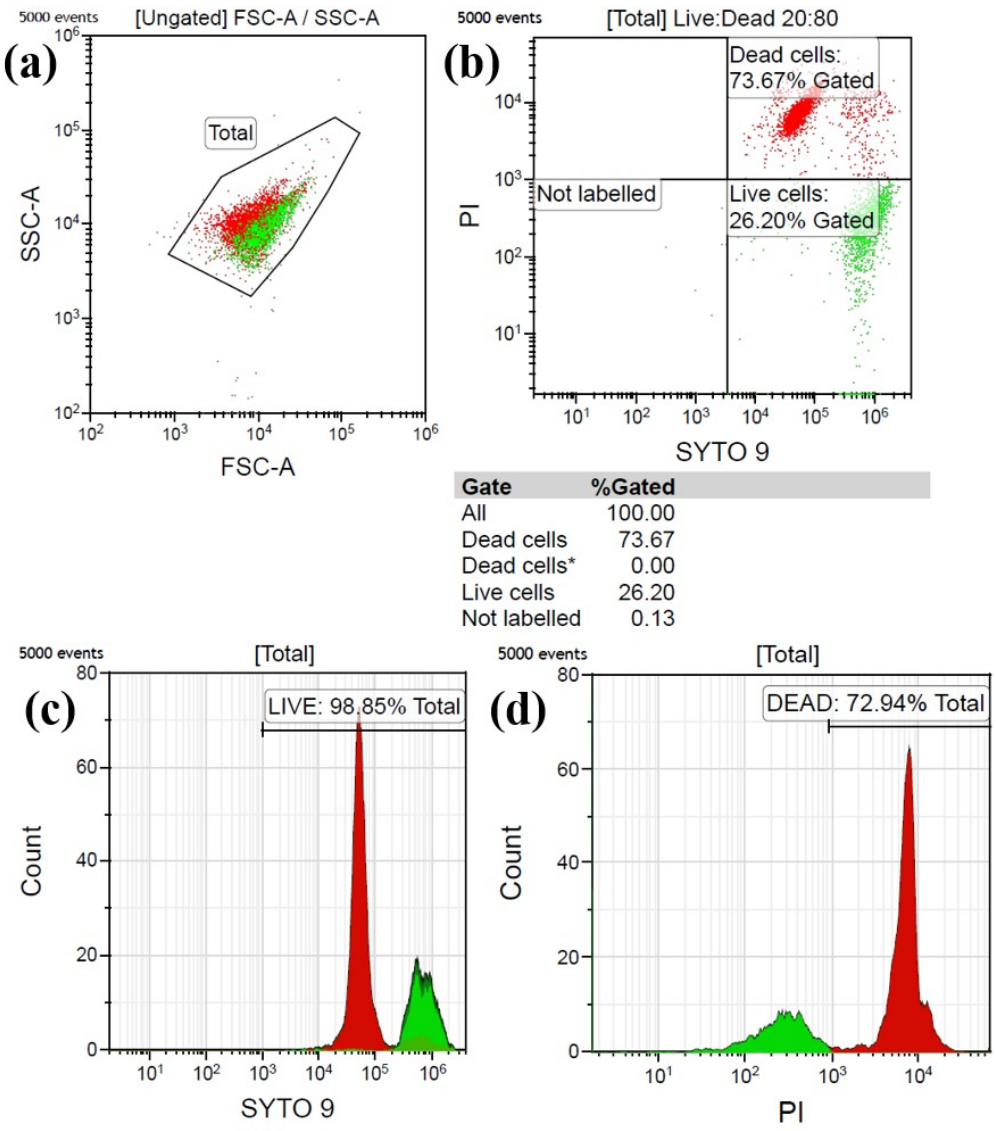



**Figure S3.** Representative cytograms for *R. capsulatus* (cells suspended in TBS). Live and dead cells were mixed at 20:80 ratio, stained with SYTO 9 and PI dyes, and assayed by flow cytometer. (a) SSC versus FSC cytogram shows a population of interest (total population of cells is gated out; *Total*). (b) PI versus SYTO 9 cytogram shows population of live and dead cells (green and red, respectively). (c, d) The number of cells stained by SYTO 9 and PI dyes, respectively. The histogram is just a different graphical representation of the Graph (b).

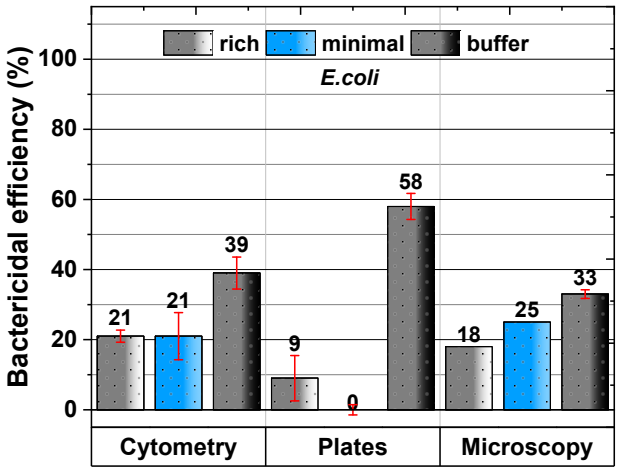
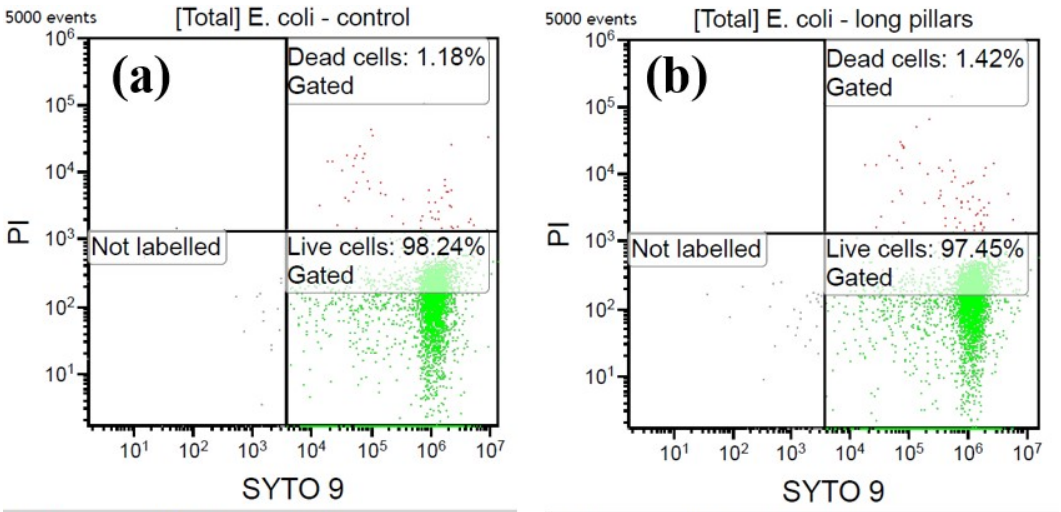


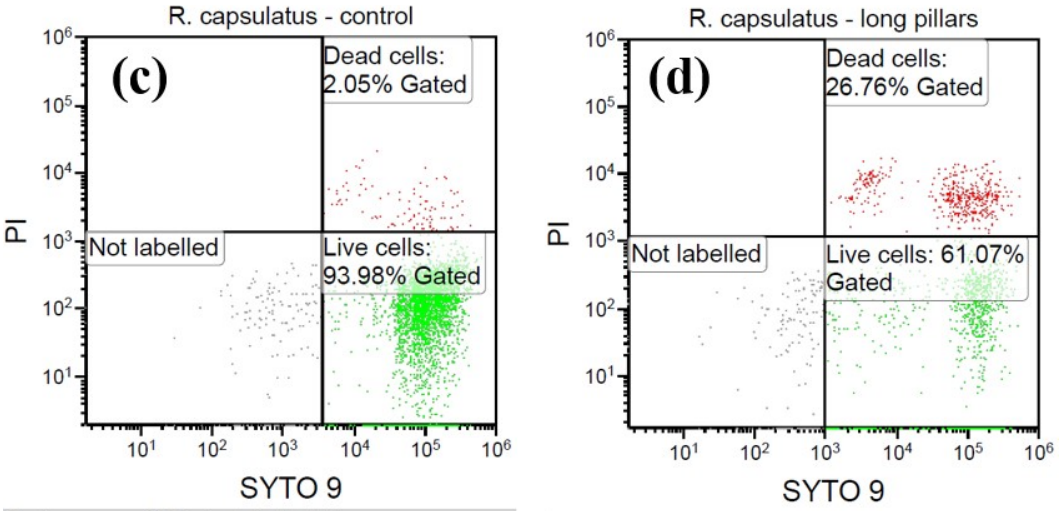

**Figure S4.** Bactericidal efficiencies of nanostructured silicon (L=390 nm, blunt tips) against *E. coli*. Bacteria were interacting with the surfaces for 2 h in rich and minimal media, and nutrient-free buffer. The results show similar results obtained in rich and minimal media with the augmented values determined in PBS buffer. The values are expressed as a mean ± SEM (n ≥ 3 independent experiments).




| Gate | %Gated | Cells/µL |
|---|---|---|
| All | 100.00 | 822 |
| Dead cells | 1.18 | 10 |
| Dead cells* | 0.02 | 0 |
| Live cells | 98.24 | 808 |
| Not labelled | 0.57 | 5 |




| Gate | %Gated | Cells/µL |
|---|---|---|
| All | 100.00 | 532 |
| Dead cells | 1.42 | 8 |
| Dead cells* | 0.00 | 0 |
| Live cells | 97.45 | 519 |
| Not labelled | 1.13 | 6 |

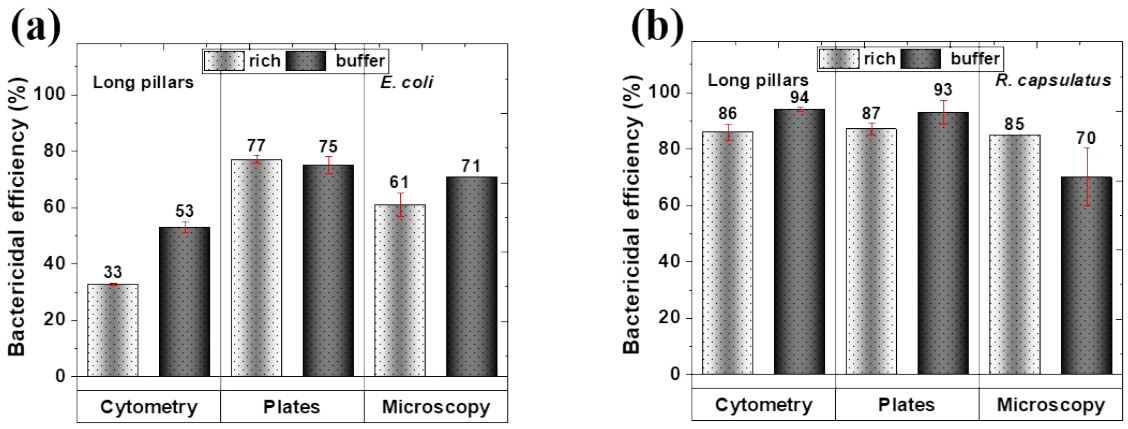



| Gate | %Gated | Cells/µL |
|---|---|---|
| All | 100.00 | 967 |
| Dead cells | 2.05 | 20 |
| Dead cells* | 0.00 | 0 |
| Live cells | 93.98 | 908 |
| Not labelled | 3.97 | 38 |




| Gate | %Gated | Cells/µL |
|---|---|---|
| All | 100.00 | 81 |
| Dead cells | 26.76 | 22 |
| Dead cells* | 0.00 | 0 |
| Live cells | 61.07 | 50 |
| Not labelled | 12.18 | 10 |

**Figure S5.** Representative cytograms of *E. coli* (a-b) and *R. capsulatus* (c-d) after 2 h of interaction with the controls (a, c) and sharp pillars (b, d). Cells were suspended in nutrient-rich media. The percentage of live and dead cell populations was determined as well as cell concentrations.




| *E. coli* | | 2 h | 4 h |
|---|---|---|---|
| **Cytometry** | **Rich** | 38% | 33% |
| | **Buffer** | 51% | 53% |
| **Plates** | **Rich** | 57% | 77% |
| | **Buffer** | 79% | 75% |
| **Microscopy** | **Rich** | 29% | 61% |
| | **Buffer** | 55% | 71% |

| *R. capsulatus* | | 2 h | 4 h |
|---|---|---|---|
| **Cytometry** | **Rich** | 87% | 86% |
| | **Buffer** | 86% | 94% |
| **Plates** | **Rich** | 85% | 87% |
| | **Buffer** | 82% | 93% |
| **Microscopy** | **Rich** | 69% | 85% |
| | **Buffer** | 25% | 70% |

**Figure S6.** Bactericidal efficiencies of etched silicon (L=3.6 µm, sharp tips) against (a) *E. coli* and (b) *R. capsulatus* with corresponding tables below the graphs summarizing the data obtained after 2 and 4 h. On the graphs, bacteria were interacting with the surface for 4 h in rich medium and nutrient-free buffers. Bactericidal efficiencies were determined based on plating method, flow cytometry, and microscopy. The values are expressed as a mean ± SEM (n ≥3 independent experiments).